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(54) Title: HYPERSENSITIVE RESPONSE ELICITOR FROM *XANTHOMONAS CAMPESTRIS*

(57) **Abstract**

The present invention is directed to an isolated *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide. The hypersensitive response elicitor proteins or polypeptides in accordance with the present invention and the isolated DNA molecules that encode them have the following activities: imparting disease resistance to plants, enhancing plant growth, and/or to controlling insects on plants. This can be achieved by applying the hypersensitive response elicitor in a non–infectious form to plants or plant seed under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a DNA molecule encoding the elicitor can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

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HYPERSENSITIVE RESPONSE ELICITOR FROM *XANTHOMONAS CAMPESTRIS*

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/103,124, filed October 5, 1998.

FIELD OF THE INVENTION

The present invention relates to a *Xanthomonas campestris* from hypersensitive response elicitor.

BACKGROUND OF THE INVENTION

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development, and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur. During incompatible interactions, bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly, Z., "Defenses Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed. Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149-177 in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations ($\geq 10^7$ cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic Pseudomonads," Nature 199:299-300; Klement, et al.,

"Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf," Phytopathology 54:474-477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," Phytopathology 64:885-890 (1974); Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in Gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., "*hrp* Genes of Phytopathogenic Bacteria," pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangl, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia, Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," Trends Microbiol. 1:175-180 (1993)). In *E. amylovora, P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), Wei,

Z.-M., et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora*, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-53 (1994)). However, *P. solanacearum popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among Gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "*Erwinia chrysanthemi* $Harpin_{Ech}$: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp*N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

The present invention identifies yet another hypersensitive response elicitor protein or polypeptide.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide.

The hypersensitive response elicitors according to the present invention have the following activity when utilized in conjunction with plants: imparting disease resistance to plants, enhancing plant growth, and/or controlling insects. This involves applying the hypersensitive response elicitor in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

As an alternative to applying the hypersensitive response elicitor to plants or plant seeds in order to impart disease resistance, to enhance plant growth, and/or to control insects on plants, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide in accordance with the present invention and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects in the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the DNA molecule encoding such a hypersensitive response elicitor can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an isolated *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide.

The hypersensitive response elicitors according to the present invention have the following activity when utilized in conjunction with plants: imparting disease resistance to plants, enhancing plant growth and/or controlling

insects. This involves applying the hypersensitive response elicitor in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

As an alternative to applying the hypersensitive response elicitor to plants or plant seeds in order to impart disease resistance, to enhance plant growth, and/or to control insects on plants, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide in accordance with the present invention and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects in the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the DNA molecule encoding such a hypersensitive response elicitor can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

The hypersensitive response elicitor polypeptide or protein derived from *Xanthomonas campestris* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

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Met Asp Gly Ile Gly Asn His Phe Ser Asn
 1               5                  10
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This hypersensitive response elicitor polypeptide or protein has a molecular weight of 13-15 kDa.

Fragments of the above hypersensitive response elicitor polypeptides or proteins are encompassed by the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding a known elicitor protein are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

The hypersensitive response elicitor of the present invention is preferably in isolated form (i.e. separated from its host *Xantamonas campestris*) and more preferably produced in purified form (preferably at least about 60%, more preferably 80%, pure) by conventional techniques. Typically, the hypersensitive response elicitor of the present invention is produced but not secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is secreted into growth medium. In the case of unsecreted protein, to isolate the protein hypersensitive response elicitor, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical

treatment. and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to heat treatment and the hypersensitive response elicitor is separated by centrifugation. The supernatant fraction containing the hypersensitive response elicitor is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the fragment. If necessary, the protein fraction may be further purified by ion exchange or HPLC.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced

into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct

positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promotor, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-

ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention further relates to methods of imparting disease resistance to plants, enhancing plant growth, and/or effecting insect control for plants. These methods involve applying the hypersensitive response elicitor polypeptide or protein of the present invention, in a non-infectious form to all or part of a plant or a plant seed under conditions effective for the elicitor to impart disease resistance, impart stress resistance, enhance growth, and/or control insects. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to impart stress resistance, to enhance plant growth, and/or to effect insect control.

As an alternative to applying the hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, and/or to control insects on the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding the hypersensitive response elicitor polypeptide or protein, which fragment elicits a hypersensitive response, and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding the hypersensitive response elicitor polypeptide or protein of the present invention can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of the isolated hypersensitive response elicitor or 2) application of bacteria which do not cause disease and are transformed with a gene encoding the elicitor. In the latter embodiment, the elicitor can be applied to plants or plant seeds by applying bacteria containing the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein. Such bacteria must be capable of secreting or exporting the elicitor so that the elicitor can contact plant or plant seeds cells. In these embodiments, the elicitor is produced by the bacteria *in planta* or on seeds or just prior to introduction of the bacteria to the plants or plant seeds.

The methods of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, and/or control insects. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases, treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of

pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solancearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthamonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops to be grown in areas where short growing seasons would otherwise preclude their growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants, preventing insects from colonizing host plants, preventing colonizing insects from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on

over 200 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm), pepper maggot, tomato pinworm, and maggots. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings), etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds or propagules (e.g., cuttings), in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide, in accordance with present invention, can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the elicitor with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to impart disease resistance to plants, to enhance plant growth, and/or to control insects on the plants.

The hypersensitive response elicitor polypeptide or protein, in accordance with the present invention, can be applied to plants or plant seeds alone or in a mixture with other materials. Alternatively, the elicitor can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains the hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM of the elicitor.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof. Suitable fertilizers include $(NH_4)_2NO_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, the hypersensitive response elicitor of the present invention need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding the elicitor are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the

outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the Gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response elicitor resulting in disease resistance, enhanced plant growth, and/or control of insects on the plant. Alternatively,

transgenic seeds or propagules (e.g., cuttings) are recovered from the transgenic plants. The seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, and/or to control insects. While not wishing to be bound by theory, such disease resistance, growth enhancement, and/or insect control may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which the hypersensitive response elicitor in accordance with the present invention is applied. These other materials, including the hypersensitive response elicitor in accordance with the present invention, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor in accordance with the present invention to impart disease resistance, enhance growth, and/or control insects. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

EXAMPLES

Example 1 - Culture Growth

For the purpose of this study, *Xanthomonas campestris pelargonii (Xcp)* was grown on Luria agar plates. From these plates, colonies were transferred to inoculate *Xcp* seed cultures. The seed cultures were grown in 250ml baffled flasks containing 50ml of 50% Luria broth. The seed cultures were grown at approximately 27°C, while shaking at 250rpm, until an optical density (λ620) of 0.5 to 0.8 was reached.

supernatant was discarded and the resulting cell pellet was resuspended in the already prepared minimal media, in a manner such that no Luria broth was introduced into the minimal media culture. A 1:10 ratio of seed culture to minimal media was consistently used for innoculating the minimal media. In other words, the cell pellet formed a 50ml seed culture which was used to inoculate 500ml of minimal media. The minimal media culture was grown in a 2.8L Fernbach flask containing 500ml of media at approximately 27°C which was agitated at 250 rpm until an optical density (λ620) of 1.7 to 2.0 was reached.

After the flask production of culture had been optimized, fermentation was transferred to 10L MicroFerm fermenter (New Brunswick Scientific, Edison, New Jersey, USA). For the 10L fermentation, the seed culture to minimal media ratio, as described above, was maintained. The fermentation was run at approximately 27°C with an initial pH of 6.0 and final pH of 5.8. The vessel was agitated at 400 rpm with 0.8 to 1.0 vessel volumes of air per minute.

A 1L 10x stock solution of the minimal media contained 39.2g of K_2HPO_4, 71.5g KH_2PO_4, 10.0g of $(NH_4)_2SO_4$, 3.5g of M_gCl_2, 1.0g of NaCl, and 34.23g of sucrose with a final pH of 6.0 to 6.2 was prepared after thoroughly mixing. The stock was sterile filtered and kept at 4°C.

Example 2 – Cell-Free Elicitor Preparation

The first step in the purification of the *Xcp* hypersensitive response elicitor was the development of a cell-free elicitor preparation ("CFEP"). The CFEP production involves four steps as described below.

1. Initial Centrifugation

In the initial centrifugation step, the minimal media cell culture was divided into 370ml aliquots and centrifuged in 500ml centrifuge bottles for 10 minutes, at 4°C, and at 8,000 rpm. The resulting cell pellet was resuspended at a 1:10 weight to volume ratio using lysis buffer. The lysis buffer consisted of 10mM NaCl and 20mM Tris-HCL at pH 8.0. Resuspension of the cell pellet was achieved

by vortexing individual centrifuge bottles. In the case of the 10L fermentation, a homogenizer was used.

2. Sonication

The resuspended pellet was then sonicated in 50ml aliquots at a setting of 5 for 3 minutes (with the horn tip, VirTris, VirSonic). While sonicating, the beaker containing the solution was immersed in an ice water bath. The resulting sonicate was kept on ice until all of the solution was sonicated. The setting used for sonication was the manufacturer's suggested maximum setting for the tip used.

3. Heat Treatment

The sonicate was placed on a preheated stir plate and brought to a rolling boil. The rolling boil was sustained for 5 minutes. After the 5 minute heat treatment, the solution was immediately placed in an ice water bath and cooled to approximately 10°C.

4. Final Centrifugation

Prior to final centrifuging, the cooled solution was brought back to its original volume with deionized H_2O, replacing the volume lost to evaporation during boiling. The solution was then centrifuged in 50ml centrifuge bottles for 30 minutes at 4°C and 15,000 rpm. The resulting supernatant from each bottle was combined and frozen in a –80°C freezer. A 1ml sample was also saved and used for testing hypersensitive response activity.

Example 3 - Protein Verification

To determine whether the hypersensitive response elicitor was indeed a protein, protease digestions were performed with CFEP. CFEP, prepared as previously described, was inoculated with protease K at a concentration of 2mg/ml. After a 1.5h incubation at 37°C, the protease inoculated CFEP along with the positive control (CFEP alone) and negative control (protease K at 2mg/ml in lysis buffer) were infiltrated into tobacco plants for hypersensitive response elicitor testing. The

positive control showed hypersensitive response necrosis, while the negative control showed no signs of testing. The protease K inoculated CFEP also showed no signs of hypersensitive response, indicating the *Xcp* hypersensitive response elicitor was sensitive to protease digestion and indeed a protein. This experiment was repeated several times with different batches of CFEP, each time with the same results.

Example 4 - Chromatographic Purification

The production of a hypersensitive response elicitor CFEP was the first step in the purification scheme for the *Xcp* hypersensitive response elicitor. Further purification of the elicitor consisted of four chromatographic steps. Anion exchange, cation exchange, various types of affinity chromatography, hydrophobic interaction, and reversed phase chromatography media were all analyzed for their utility in purifying the hypersensitive response elicitor. All chromatography experiments were conducted with the FPLC and FPLC detector (Pharmacia Biotech, Upsala, Sweden). The final purification scheme used consisted primarily of chromatography media that binded to the *Xcp* hypersensitive response elicitor based the hypersensitive response elicitor's hydrophobic characteristics.

1. Butyl Sepharose

The CFEP was first bound to a medium strength hydrophobic interaction chromatography medium. The CFEP was adjusted to 600mM NaCl and loaded on to a Butyl Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with a 75-100% buffer B gradient. Buffer A contained 600mM NaCl, 20mM Tris-HC1 at pH 8, and buffer B contained 10mM Tris-HC1 at pH 8. At 85% B gradient, buffer B was exchanged for deionized H_2O.

2. Mono S

The fractions judged to have the highest concentration of the hypersensitive response elicitor (determined by making hypersensitive response dilution series with active fractions) from the Butyl Sepharose column were pooled together and loaded on to a strong cation exchanger, Mono S (Mono S 10/10 column,

Pharmacia Biotech). Prior to loading, the pooled fractions were adjusted to 20mM NaCl, 20mM Tris-HCl at pH 5.5. Buffer A was 20mM NaCl, 20mM Tris-HCl at ph 5.5. Buffer B contained 1M NaCl, 20mM Tris-HCl at pH 5.5. The sample was loaded, followed by a 0% buffer B wash and then 100% buffer B wash. The hypersensitive response elicitor did not bind to the Mono S medium, but, at pH 5.5, many of the contaminates in the sample did. Thus, it served as a non-binding chromatography. Immediately following the collection of the flow through (the hypersensitive response elicitor fraction), the solution's pH was adjusted to 8.0.

3. Phenyl Sepharose (low)

The active fraction from the Mono S column was then loaded on to a Phenyl Sepharose 6 Fast Flow low substitution (a weak hydrophobic interaction medium, Pharmacia Biotech, Uppsala, Sweden). Buffer A contained 1M NaCl, 20mM Tris-HCl at pH 8, and buffer B contained 10mM Tris-HCl at pH 8.0. As mentioned previously, at 85% B, buffer B was changed to deionized H_2O. The hypersensitive response elicitor fraction was eluted as the gradient reached 100%B. The fractions which were then used in the next purification step contained the highest concentration of the hypersensitive response elicitor. Since the active fraction eluted in deionized H_2O, it was necessary to stabilize the fraction by adjusting them to 20mM NaCl and 20mM Tris-HCl at pH 8.0.

4. Reversed Phase Chromatography

The final chromatographic step in the purification of the hypersensitive response elicitor utilized a ProRPC 5/5, reversed phase column (Pharmacia Biotech, Uppsala, Sweden). The sample was adjusted to 15% acetonitrile (HPLC grade) and 0.1% TFA (HPLC grade) and loaded at a flow rate of 0.7ml/min. After a 5ml wash, a 15-50% B gradient was run over 58ml at a flow rate of 0.7ml/min. 1ml fractions were collected for the entire volume of the gradient. The *Xcp* hypersensitive response elicitor eluted at approximately 25%B. Fractions containing the hypersensitive response elicitor were determined by visual identification on a silver stained PAGE-SDS gels. After visual identification, fractions that were relatively pure and contained

mentioned lysis buffer to remove the acetonitrile and TFA. The dialyzed fraction was then concentrated approximately 100 fold using a Centricon 3,000 MWCO (Amicon, Beverly, MA).

Example 5 - Electroelution of the *Xcp* Hypersensitive Response Elicitor

The final concentrate was then run on a hand poured 18% acrylimide PAGE-SDS mini gel (gel was poured in an empty Novex gel cassette and run with a Novex X-Cell apparatus, San Diego, CA). The gel was stained and destained with normal Coomassie Blue staining techniques. Because of extreme overloading of the gel, it was possible to see and cut out the band that correlated with the hypersensitive response elicitor. The cut out gel was then loaded into an Elutrap (Schleicher & Schuell, Keene, New Hampshire). The protein present on the cut out was eluted off the gel, and into a collection chamber. A non-SDS tank buffer was used in the Elutrap to produce a sample relatively free of SDS. A portion of the resulting fraction was then run on a 16% acrylimide PAGE-SDS mini gel (Novex gel and apparatus). The gel was silver stained in order to determine the sample's purity. In addition, a portion of the eluted fraction was used to make hypersensitive response elicitor dilutions to determine the relative concentration of hypersensitive response elicitor present in the final sample.

Example 6 - Visualization of the Hypersensitive Response Elicitor

Perhaps the most significant and troublesome characteristic encountered during the isolation of the *Xcp* hypersensitive response elicitor was its abnormal staining characteristics. PAGE-SDS gels that had been stained and destained using normal Coomassie Blue techniques showed the hypersensitive response elicitor band only when the gel was extremely overloaded. Even under overloaded conditions, the elicitor stained very faintly and to some extent temporarily. Complete destaining of the gel usually resulted in fading of the hypersensitive response elicitor band. Only with silver staining techniques was it possible to visualize the elicitor at a seemingly lower concentration. The band, depending of the concentration of the hypersensitive response elicitor present in the sample, either

appeared as a negatively stained band with relatively distinct borders (at higher concentration) or as a slightly off-colored band, almost resembling a shadow with non-distinct, fuzzy borders. Silver stained PAGE-SDS gels were used to visualize the hypersensitive response elicitor in all cases, except when the gel was to be used for electroelution with the Elutrap or protein transfer on to a PVDF membrane.

As in the case of Coomassie Blue staining of PAGE-SDS gels, Coomassie Blue staining of PVDF membranes (used to immobilize the protein for N-terminal sequencing) resulted in very faint staining if intensely overloaded. Normal loading volumes resulted in a seemingly clean membrane with no apparent protein bands.

Example 7 - N-terminal Sequence of the *Xcp* Hypersensitive Response Elicitor

After purification of the elicitor had been achieved it was then possible to learn the N-terminal sequence of the protein. The purified *Xcp* hypersensitive response elicitor protein was run on a SDS-PAGE. From this gel, the protein was transferred to a PVDF membrane via a Trans-Blot (Bio-Rad, Hercules, CA). The membrane containing the protein was subjected to an automated Edmans Degradation process followed by high-pressure liquid chromatography for detection and identification of the individual amino acids. The first ten amino acids of the *Xcp* hypersensitive response elicitor were determined to be:

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Met Asp Gly Ile Gly Asn His Phe Ser Asn
 1               5                  10
```

(SEQ. ID. No. 1).

Example 8 - Elicitor-Induced Disease Resistance in Tobacco and Tomato

The hypersensitive response elicitor of *Xanthomonas campestris pv. pelargonii* was sprayed on tobacco plants at a concentration of approximately 5 ppm. Three days after the treatment, the plants were inoculated with tobacco mosaic virus (TMV) at a concentration of ca. 2 ppm. Four days after inoculation, the elicitor treated plants showed more than a 65% reduction in the number of lesions compared

to untreated control plants. In addition to resistance to TMV, the hypersensitive response elicitor also induced resistance to bacterial wilt of tomato.

Example 9 - The Hypersensitive Response Elicitor-Induced Growth Enhancement of Tomato

To demonstrate that the hypersensitive response elicitor of *Xanthomonas campestris pv. pelargonii* can enhance plant growth, tomato seeds were soaked in an elicitor solution of ca. 5 ppm for more than 4 hours. The seeds soaked in the same solution but without the elicitor protein were used as untreated control. Both the treated and untreated seeds were planted in eight inch pots with artificial soil, 20 seeds per pot. Twenty days after planting, the size of the tomato seedlings were measured. The plants derived from the elicitor treated seeds were 10% fuller than those generated from the untreated seeds.

A proteinaceous hypersensitive response elicitor has been purified to near homogeneity from the plant pathogen *Xanthomonas campestris pelargonii*. The protein has a molecular weight of approximately 14kDa and is heat tolerant. The hypersensitive response elicitor exhibits unusual staining characteristics when stained with normal Coomassie Blue and silver staining techniques. The *Xanthomonas campestris pelargonii* hypersensitive response elicitor is a member of the hypersensitive response elicitor protein family due to its shared characteristics with other proteins of this family. These characteristics include biochemical characteristics, the ability to elicit a hypersensitive response, and, from preliminary experiments, the ability to induce disease resistance and plant growth enhancement.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

WHAT IS CLAIMED:

1. An isolated *Xanthomonas campestris* hypersensitive response elicitor protein or polypeptide.

2. An isolated hypersensitive response elicitor protein or polypeptide according to claim 1, wherein the protein or polypeptide has a molecular weight of 13-15 kDa.

3. An isolated hypersensitive response elicitor protein or polypeptide according to claim 1, wherein the protein or polypeptide has an amino acid sequence of SEQ. ID. No. 1.

4. A method of imparting disease resistance to plants comprising:

applying a hypersensitive response elicitor protein or polypeptide according to claim 1 in a non-infectious form to a plant or plant seed under conditions effective to impart disease resistance.

5. A method according to claim 4, wherein plants are treated during said applying.

6. A method according to claim 4, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the hypersensitive response elicitor in natural or artificial soil and

propagating plants from the seeds planted in the soil.

7. A method of enhancing plant growth comprising:

applying a hypersensitive response elicitor protein or polypeptide according to claim 1 in a non-infectious form to a plant or plant seed under conditions effective to enhance plant growth.

8. A method according to claim 7, wherein plants are treated during said applying.

9. A method according to claim 7, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the hypersensitive response elicitor in natural or artificial soil and

propagating plants from the seeds planted in the soil.

10. A method of insect control for plants comprising:

applying hypersensitive response elicitor protein or polypeptide according to claim 1 in a non-infectious form to a plant or plant seed under conditions effective to control insects.

11. A method according to claim 10, wherein plants are treated during said applying.

12. A method according to claim 10, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the hypersensitive response elicitor in natural or artificial soil and

propagating plants from the seeds planted in the soil.

SEQUENCE LISTING

<110> Eden Bioscience Corporation

<120> HYPERSENSITIVE RESPONSE ELICITOR FROM XANTHOMONAS CAMPESTRIS

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 99/23265

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/195

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
X	SWANSON, S. ET AL.: "Isolation and characterization of an HR elicitor from Xanthomonas campestris." PHYTOPATHOLOGY, vol. 88, no. 9 SUPPL., September 1998 (1998-09), page S87 XP000866309	1-3
Y	the whole document	4-12

X	WO 98 24297 A (CORNELL RES FOUNDATION INC) 11 June 1998 (1998-06-11)	1
Y	page 12, line 16 -page 13, line 25 page 25, line 39 -page 26, line 16 abstract	4-6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents. such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 2000	20/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office. P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk	

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/23265

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 32844 A (CORNELL RES FOUNDATION INC) 30 July 1998 (1998-07-30) page 9, line 3 -page 10, line 7	1
Y	page 22, line 9 - line 37 abstract	7-9
X	WO 98 37752 A (CORNELL RES FOUNDATION INC) 3 September 1998 (1998-09-03)	1
Y	page 3, line 27 -page 4, line 29 page 16, line 9 - line 37	10-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. ıal Application No
PCT/US 99/23265

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WO 9837752 A	03-09-1998	AU 6666498 A	18-09-1998